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VITAL STAINING AS EVIDENCE FOR WING CIRCULATION IN THE CABBAGE BUTTERFLY *PIERIS RAPAE*

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INTRODUCTION

A BRIEF REVIEW OF THE LITERATURE on wing circulation is necessary to introduce the present work.

1. *Hemolymph in the Wing.* — The question of wing circulation obviously presupposes the presence of hemolymph, on which point there may be popular misconception. Lutz (1935), in his well-known "Field Book of Insects", asserted that "Wing 'veins' or 'nerves' neither contain blood nor have anything to do with the nervous system". In fact, innumerable studies report the presence of hemolymph in the veins of insect wings, simply because this observation is made easily by crushing or cutting the veins under a low-power microscope.

2. *Wing Circulation in Insects in General.* — According to Yeager and Hendrickson (1934), wing circulation was reported for a grasshopper in 1744 and up to 1934 had been claimed for 50 species representing 10 orders. The Lepidoptera in this list are *Smerinthus populi* and *Sphinx convovulvi.* Circulation in the cockroach wing is easily observed and has been the subject of a number of studies, e.g. by Yeager and Hendrickson (1934) and Clare and Tauber (1942). General accounts of insect physiology by Beard (1953) and Wigglesworth (1965) conclude that wing circulation probably is universal. However, wing circulation is far from clearly established in Lepidoptera.

3. *Wing Circulation in Lepidoptera.* — Reports range from denial of circulation to the assumption that it exists as part of respiration by the wing. Zeller (1938) claimed that there is no circulation in the adult wing of the meal moth *Ephestia kühni-*

ella. Holland (1931) left open the possibility of circulation by saying, "The wings consist of a framework of horny tubes which are in reality double, the inner tube being filled with air, the outer tube with blood. . . . After emergence, the circulation of blood in the outer tube is largely, if not altogether, suspended". Assuming circulation, Packard (1880) stated that "the aeration of the blood is carried on in the wings, and thus they serve the double purpose of lungs and organs of flight". This resembles the conclusion (Portier, 1932a, Portier and Emmanuel, 1932) that blood and air circulation proceed in the wings and that the sunlight "acts as a motor" by creating temperature differentials. Another suggestion by Portier (1932b) is that the seemingly hollow scales and peduncles of *Parnassius* wings are permeable to gases and communicate with tracheal capillaries.

Clare (1965) studied various Lepidoptera and in no case was able to detect an active general circulation, although evidence suggested that circulation occurs at least in recently-eclosed adults. Clare concluded that in the Lepidoptera "the problem of wing circulation is far from satisfactorily understood" and "remains in an unsatisfactory state of affairs". Further references to Clare's work will be made in the discussion.

EXPERIMENTAL

1. *Dye Feeding Procedure.* — Adults of *Pieris rapae* (L.) were fed an aqueous solution of 10% sucrose and 1% neutral red, this dye being chosen because of its efficacy in vital staining of *P. rapae* when fed to larvae (Kolyer, 1965). An 11.2% sucrose solution was used for feeding *Pieris brassicae* by David and Gardiner (1961), and insects have been observed to accept sugar solutions containing neutral red or other dyes (Stober, 1927).

The feeding procedure was that recommended by Urquhart (1960) for *Danaus plexippus.* That is, a small piece of cotton wool was placed in a watchglass and saturated with the solution, and the butterfly was placed on this with wings held together over the back between thumb and forefinger. In this procedure, the tarsi touch the sugar solution, which contact is claimed to stimulate feeding in *Pieris* (work of Minnich as cited by Wigglesworth, 1965), although Verlaine (1927) concluded that *P. rapae* tastes with the tongue rather than the tarsi, and Anderson (1932) found questionable discrimination of sugar by the legs of *P. rapae.*

At the end of feeding, to avoid damaging the legs, which tend to catch in the fibers, a pencil point was offered the insect so that it voluntarily climbed off the cotton. The butterflies were kept in a dark box between feedings to minimize wing damage by flight.

In order to conserve dye while always having fresh (less than one week old) sucrose solution, separate solutions in deionized water were prepared. These were 2.0 wt.-% neutral red (Matheson Coleman & Bell Div., Matheson Co., 88% total dye content), shaken occasionally and allowed to stand for several days to ensure complete dissolution of the dye, and 20 wt.-% sucrose. Before each feeding, equal volumes of the solutions (5 drops each) were mixed in a watchglass before adding just enough cotton to absorb most of the liquid.

2. *Dye Feeding Experiments.* — In the first experiment, five females, eclosed approximately 10 hours before, were given their first feeding by the above method. The proboscis was not extended voluntarily but had to be uncoiled with a pin and forced into the solution, where it then was kept voluntarily for under 2 minutes. At the end of two days, on the 5th feeding, the proboscis was extended voluntrily by two individuals and was kept in the solution for up to 2.5 minutes. Pink color was apparent in the wing veins, especially near the body. At the end of 4 days, on the 10th feeding, the butterflies could be left sitting on the cotton without restraining the wings, and the longest feeding time was 10 minutes.

At 5.5 days, four of the females were placed in a cage with two unstained males (38-48 hours old) and exposed to sunlight for about 10 hours. Thirteen eggs, ranging from pale to deep pink, were found on cabbage leaves which had been hung up in the cage. The females were fed again (13.5 minutes maximum feeding time), and the males were fed 10% sucrose solution without neutral red. Exposure to sunlight for two more days yielded 13 more pink eggs. All the eggs collapsed and shriveled within three days. Dissection indicated that the pink color was concentrated in the shell, which contained turbid, pale-yellow liquid.

At this point two of the females died. Examination under 16X showed red proboscis, eyes approximately normal green (which turned to rich brown-red within two days after death vs. brown for unstained specimens), interior of abdomen purplish red, and interior of thorax orange.

Fig. 1. Specimen of *Pieris rapae* (♀) showing vital staining of the wing veins by feeding neutral red and sucrose solution for 10 days (details in text).
Fig. 2. Specimen of *P. rapae* (♂) fed neutral red and sucrose solution for 8 days (details in text). The photograph was taken through a 16X microscope.

Fig. 3. End view of a section of a hindwing from a specimen of *P. rapae* (♀) whose body, with ventral portion of thorax amputated, had been immersed for one hour in 2% neutral red solution. The photograph was taken through a 100X microscope.
Fig. 4. Apex of a forewing from a specimen of *P. rapae* (♂) which had been immersed in neutral red solution (in the manner described for Fig. 3) for 15 minutes. The photograph was taken through a 16X microscope.

The remaining two females were fed three more times. On the final feeding (10 days from the start of the experiment) both extended the proboscis voluntarily and drank the solution for 25 and 33 minutes. After two more days both seemed near death (movements were feeble) and were killed and spread. One specimen is shown in Figure 1.

In the second experiment, the wings of two males, eclosed about 20 hours before ,were mutilated on the righthand side; in one the margins of both fore- and hindwings were trimmed off, and in the other the apex of the forewing was removed. A third (undamaged) male also was included. These were fed 14 times in 8 days, at the end of which time, still capable of vigorous flight, they were killed and spread. As with the females, feeding time tended to increase with age (maximum 17 minutes, at 5 days), and the proboscis often was extended voluntarily. Again, pink color appeared in the veins, and the eyes turned rich brown-red after death (see Figure 2). The antenna clubs were pink to red.

During the feeding experiments, room conditions were 71-78°F and 32-38% relative humidity.

3. *Injection of Dye.* — A capillary was used, in the manner of Campbell (1932), to inject approx. 1.7 milligrams of 2% neutral red solution into the thorax through the dorsal surface of four individuals 25-35 hours old. The liquid was forced in by lung power, via a rubber tube attached to the capillary. Puncturing the integument of many insects results in no hemorrhage (Beard, 1953), and this was true in the present case.

Red color appeared in the annular channels of the veins. In a somewhat similar experiment, Muenchberg (1966) injected $Na_2S^{35}O_4$ into dragonflies to gain evidence for wing circulation. In the present work no additional attention was paid injection because vital staining gave the same effect.

4. *Dissection Methods.* — In one procedure, the lower part of the thorax, including the legs, of a living butterfly was clipped off with small scissors just before immersing the underside of the body in 2% neutral red solution. Rapid movement of the dye into the veins was noted (see Results and Discussion). Similar dye movement was noted by the alternate procedure of severing a wing in the basal region and inserting the cut edge in the dye solution, but this method was not so convenient as the first.

5. *Technique for Observing Hemolymph in Veins.* — To observe liquid in the veins, the lefthand wings were clipped off at

the base, and the body was fastened with transparent tape to a microslide so that the righthand wings were lying flat. Hemolymph (violet to pink for the vitally-stained veins) was observed easily at 16X by rubbing away the scales and running the edge of a needle point along the vein to collapse it and concentrate a drop of liquid inside the trachea. For closer observation, Permount mounting medium (Fisher Scientific Co.) was dropped on the slide under the wing and then on top of the wing, and a cover glass was added. Due to refractive index similarity, the veins in the living wing then could be observed by transmitted light at 100 or 430X.

6. *Experiment on Respiration by Wings.* — In an incidental experiment, the wings of a living butterfly were cut off about a third of the way from the base and were inserted through a slot in a rubber sheet covering a jar with a little carbon tetrachloride in the bottom. After two hours the insect was struggling actively, suggesting that wing tracheae play little, if any, role in general respiration.

RESULTS AND DISCUSSION

1. *Observation of Hemolymph in the Veins.* — With the living wing of a 26-36 hour old male in Permount to give transparency (see Experimental) the hemolymph was observed easily by transmitted light (substage illuminator), but hemocytes seemed essentially absent. By intensive study at 430X, a few small particles were believed to have been seen (but motes in the eye can create this illusion) in the veins in the discal area of the forewing moving toward the margin at 0.7-1.3 mm/min. Tauber and Snipes (1936) report an average velocity of 34 mm/min., with 10 mm/min. as the lowest velocity, for hemocytes in the elytron of the cockroach.

Application of pressure to the thorax caused many colorless bodies to appear, apparently by dislodgment from the walls of the channels. Clare (1965) reported such dislodgment of "fatty-like tissue cells" when the amputated wings of Lepidoptera were pressed between microslides. These bodies could be seen "rounding the bend" at the extremities of the veins into the marginal channel. Relief of the pressure allowed the bodies to drift back toward the base of the wing.

These observations show that hemolymph existed throughout the vein system of a 26-36 hour old butterfly and that this hemo-

lymph communicated with the thorax, but circulation was not detected.

2. *Vital Staining Results.* — The red color of the dye appeared in all the veins of the dye-fed specimens to within a few millimeters of the margin (see Figure 1). Even when the margins of both wings, or the apex of the forewing, had been amputated before feeding, the veins were stained to within 4 mm or less of the edge. In one female (not that shown in Fig. 1), sections of the marginal channel were well-stained in both fore- and hindwings. This result clearly demonstrates that hemolymph can exist essentially throughout the vein system of well-fed adults and that this hemolymph communicates with the hemolymph of the body, but the question of circulation must be considered carefully.

It might be proposed that the hemolymph in the wings remains stagnant and the dye molecules diffuse into it. Such diffusion was ruled out for the dissection results (see below) and is believed to be unacceptable as the explanation for vital staining of the veins, but another problem remains. Admitting that flow of dyed hemolymph into the veins is necessary to account for staining, there are two alternatives: (1) there is a return flow of hemolymph to the body (true circulation) or (2) evaporation of water from the veins and wing membrane causes fresh hemolymph to flow out from the thorax *without* a return flow (*not* true circulation). Of course, the real situation may be complex, e.g. true circulation may occur in young adults with outward flow predominating in older individuals.

3. *Dissection Results; Evidence of "Water Deficit" in Veins.* — The technique of removing the lower part of the thorax and immersing in dye solution (see Experimental) was used with wild adults of unknown age. The red solution appeared in the veins in one minute and reached the margin, in some cases, within 10 minutes. Some random observations of distance attained (from base of hindwing) vs. time are: 8 mm in 7 min., 15 mm in 5 min., and 17 mm in 9 min. For a forewing, an observation was 12 mm in 11 min. Similar movement into the channels occurred when a wing section was inserted in the dye solution.

These rates (up to 3 mm/min.) must be explained by capillary action and/or suction rather than diffusion of dye molecules into stagnant hemolymph. As evidence, a glass capillary tube, approx. 0.2 mm inside diameter, was filled with clear, green

hemolymph from the thorax of a living adult, and 2% neutral red solution was added to one end so that it contacted the hemolymph. The color front had advanced into the hemolymph a total of about 1 mm after 9 min., 2 mm after 23 min., and 3 mm after 43 min. In the much-smaller channel of the vein this advance would be, if anything, slower because of even less opportunity for convection currents to provide mixing.

The dye solution moved within the channel (annulus) of the vein, not the lumen (trachea), as shown by cross-sectioning under the microscope. Figure 3 shows an example. This is an end view of a wing section, not a microtomed slice, so that the light-red color visible in the lumen is the result of light shining through the deep-red wall-channel.

The dye solution followed various courses, seemingly random, in filling the channels of some of the veins but not others. Using the venation scheme illustrated by Klots (1951), in both hindwings from the same adult the dye solution ran out into veins R and Cu_2 *only*. In one wing the dye ran a short distance down the marginal channel from the end of R ; in the other wing the dye ran up the margin from Cu_2 and back *into* M_3 a short distance. This tendency of dye solution to travel along the margin and then run back into a vein was noted in other cases. For example, in a forewing the dye ran out the 2nd A vein only, up the margin, and back into Cu_2 a short distance. Another example is shown in Figure 4, where the dye can be seen to have run back into R_3.

Results for wings from the same adult were sometimes dissimilar. For example, in one forewing the dye followed both upper and lower veins bounding the discal cell, in the other forewing only the lower vein. In another case, the dye appeared in approximately 80% of the total venation of one forewing vs. approximately 10% of the other. In one hindwing, an estimated 95% of the total venation was colored, with "breaks" in the middle of veins where dye fronts had been moving in opposing directions.

To further demonstrate that a deficit of liquid in the channels was causing the dye solution to run along the veins, the thorax of a male, taken outdoors, was cut as usual, and the underside of the body was immersed in water. After one hour, the insect was transferred to 2% neutral red solution, and after another hour the wings were removed and examined. There was no red color in the veins, even in the basal area, indicating that water had filled the channels.

The water deficit would ve expected to be less in recently-eclosed individuals, and this was found to be true. By the usual technique (thorax cut), a 10 hour old male (not fed), after one hour in the dye solution, showed red in only one forewing (in one vein, out to 4 mm) and one hindwing (out to 2 mm in the basal veins). Similarly, a 21 hour old male (not fed) showed no red in the forewing veins and red to 4 mm in a vein on one hindwing.

CONCLUSION

Two mechanisms, which both may play a role, must be considered in attempting to explain the vital staining results: (1) the hemolymph circulates to and from the body, or (2) evaporation from the wing surface creates a gradual flow of hemolymph from the body into the vein-channels with *no* return flow.

Two observations favor the latter hypothesis:

(1) Removal of the marginal channel before dye-feeding did not prevent the red color from appearing close to the ends of the veins. According to Clare (1965), "When this (marginal) channel is broken much of the circulation may be arrested", as would be expected if the hemolymph must flow out to the margin through one vein and back to the body through another. Assuming that hemolymph cannot move from one vein to another within the two-layered wing membrane, the only way to explain a close approach of dyed hemolymph to the ends of severed, "dead-end" veins is by one-way flow or the supposition of an efferent channel *and* an afferent (return) channel within the same vein, a possibility for which there was no evidence.

(2) A "water deficit" in the vein-channels of older adults was demonstrated. The random paths followed by the dye solution in making up this deficit did not suggest that hemolymph normally flows outward in certain veins and back through others, as in the cockroach wing. In fact, the presence of a deficit in the majority of the veins, as noted in many cases, would seem to preclude active circulation whether the deficit be caused by (a) empty space in the annular channels or (b) expansion of the tracheae against the walls of the veins to close the channels. Clare (1965) states "Some aged adults may contain little or no hemolymph in the wing channels, but this study suggests that this is probably exceptional." Further, "If hemolymph (moisture) gets low in the body, perhaps some of the channels become air-filled."

The present work indicates that a moisture or hemolymph deficiency begins within one day from eclosion for unfed specimens and certainly is common among *P. rapae* in the field. A possibility is that in the vitally-stained individuals the supply of water in the body was ample so that dyed hemolymph flowed outward from the thorax, as required by evaporation, to keep the vein-channels filled and the wing, as described by Clare (1965), in a "moist and lifelike condition".

Among the questions raised by the results are: Can the water deficit be only temporary and disappear, i.e. channels become refilled, after a butterfly feeds? How long can a deficit of water in most of the channels be tolerated before the wing becomes overly desiccated? Does true circulation exist at least in new adults before the deficit becomes widespread. Information might be secured by ageing and intermittently feeding a sizeable number of individuals which had eclosed at about the same time and by taking statistically-significant samples to follow the extent of the water deficit by the dissection technique described above.

In short, vital staining seems to demonstrate efferent flow of hemolymph but does not prove the afferent flow required for true circulation. Efferent flow alone, without return of hemolymph to the body, explains the data.

SUMMARY

Beginning 10-20 hours from eclosion, adults of *Pieris rapae* (L.) were fed (for 8-10 days) an aqueous solution of 1% neutral red (an effective vital stain) and 10% sucrose. The annular hemolymph channels of the wing veins, including parts of the marginal channel, were found to contain pink (dyed) hemolymph. This result demonstrates efferent flow but leaves in question the afferent (return) flow required for true circulation. Amputation of the lower part of the thorax of living adults (taken outdoors) and insertion of the body of 2% neutral red solution caused flow (up to 3 mm/min.) of the dye solution into the vein-channels, often including the marginal channel and staining from about 10% to 95% of the total venation. This effect was slight (veins colored only in basal region) in 10 or 21 hour old individuals and was prevented entirely by placing the body in water for a time, allowing water to fill the channels, before transferring to the dye solution. The "water deficit"

presumably is created by evaporation and may involve empty (air-filled) channels and/or channels closed by expansion of tracheae. It is suggested that efferent flow alone, without true circulation, may explain the vital staining of the veins. In support of this, removal of the marginal hemolymph channels before dye-feeding would be expected to suppress true circulation (Clare, 1965), but severed, "dead-end" veins were found to be stained to within a few millimeters of their ends.

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